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Purification of membrane proteins free from conventional detergents: SMA, new polymers, new opportunities and new insights.

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Abstract

Membrane proteins remain a somewhat enigmatic group of biomolecules. On the one hand they mediate some of the most important processes in biology with molecular mechanisms that are often elegantly complex. On the other hand they are exceptionally challenging to produce, making studies of membrane protein structure and function among the most difficult projects undertaken by biochemists. The central issue with studies of a membrane protein has been the need to extract them from their native lipid environment before purification and production of a homogenous sample. Historical approaches have utilized detergent solubilisation but these often lead to a sample with low activity and stability. In the past 15 years a new approach that focuses on preserving the local lipid environment surrounding the membrane proteins has been developed. The latest, and perhaps most complete, incarnation of this method has been the use of polymers based on styrene maleic acid (SMA) to stabilise nanoscale discs of lipid that contain membrane proteins. In this review we examine the range of SMA-related polymers that have now been shown to have utility in the production of membrane proteins. We discuss the differences between the polymers and attempt to discover rules and trends that explain their behavior.

Introduction

Located at the interface between cells and their environment and on the surfaces of organelles, membrane proteins play an integral role in coordinating cellular function.

Current estimates predict that more than 50% of modern drugs target membrane proteins [1], demonstrating the importance of the study of these systems. However, the purification of membrane proteins for structural and functional study remains a challenge. Despite close to 20% of both the human and *E. coli* genome encoding for membrane proteins, they represent less than 1% of the structural entries to the Protein Data Bank (PDB) repository [2].

One of the biggest roadblocks to studying membrane proteins is the need to separate them from unrelated proteins co-localised in the same membrane continuum [3]. Historically this has been achieved by removing them from their lipid environment prior to purification. Classical 'head and tail' detergents such as dodecyl maltoside (DDM) have been used to perform the membrane extraction process. When present above a critical micelle concentration (CMC) these amphiphilic compounds disrupt the membrane to form soluble micelles and are thought to arrange around the hydrophobic transmembrane domain of membrane proteins in a torus, replacing surrounding lipids [4]. It had been assumed that the detergent micelle would be able to sufficiently replicate the lipid environment ensuring that the membrane protein retained native structure and function. While the hydrophobic interior and polar exterior of the native membrane is partially mimicked by a detergent micelle, it has become evident that the micelle acts as a poor replacement to a lipid. The composition of membranes can be highly variable, and it has been demonstrated that alteration of the chemical environment around these proteins can affect their activity [5]. Membrane proteins are also known to associate to regions of specific components, for example in lipid rafts, that influence their function [6]. For these reasons, it is clear that detergents fail to sufficiently replicate the complex environment of the cell membrane.

To address these issues a new approach to membrane protein solubilisation has been developed, which recognizes the paramount importance of phospholipids in maintaining correct folding and function of membrane proteins. These methods attempt to produce a particle that contains the chosen membrane protein whilst maintaining its local lipid environment. A number of approaches have been developed over the past 15 years to achieve this challenging goal. These include the use of peptides that stabilise bilayer fragments (membrane scaffold proteins, MSPs) [7], amphipols [8] and most recently styrene maleic acid copolymers [9]. This latter method, developed in 2009 by Knowles *et al.* has

generated significant interest in the past few years. Unlike previous methods, styrene maleic acid-mediated solubilisation enables direct extraction of the protein **complete with its native annular lipid environment** from the bulk membrane without prior detergent extraction.

Applications of SMALP technology to membrane protein studies

Styrene maleic acid (SMA) is an amphipathic, synthetic copolymer made up of hydrophilic maleic acid and hydrophobic styrene moieties, which has been recently found to have significant applications outside of its principal use in the plastics industry. Amphipathic polymers such as SMA were found to associate with and destabilise lipid bilayers in a pH-dependent manner, forming discoidal lipid-polymer assemblies [10]. This behavior was exploited to produce homogenous, membrane protein-containing particles without the need for the use of classical detergents.

Since the initial demonstration of the SMALP method for the stabilisation of a membrane protein in aqueous solution [9], SMA has been used to purify a range of important membrane proteins, including GPCRs [11], transporters [12] and ion channels [13] (For review see [14]). This is achieved by the direct addition of SMA to native membranes, removing the need for any classical 'head-and-tail' detergents in the preparation whilst keeping a near native membrane environment. Importantly, SMALP-encapsulated proteins are amenable to studies utilising common biophysical techniques such as circular dichroism (CD) spectroscopy [9], solid [15] and solution state [16] nuclear magnetic resonance spectroscopy (NMR), analytical ultracentrifugation (AUC) [9], X-ray crystallography [17] and cryo-electron microscopy (EM) [18].

The vast majority of studies using the SMA method in the production of membrane proteins have focused on the use of two related polymers. These polymers are of similar lengths and both are made using the same process, the only difference being that one contains a 2:1 ratio of styrene to maleic acid [13,19–21] while the other contains a 3:1 ratio [17,22]. These polymers seem to be very effective at solubilising a wide range of proteins but have a number of limitations, the most troublesome being that neither functions in acidic conditions or in the presence of millimolar concentrations of magnesium and calcium ions. This latter limitation in particular presents challenges in the study of proteins that

require these ions for activity (e.g ATPases). In addition it is becoming clear that in some cases encapsulation in the lipid particle leads to an inhibition of the natural conformational changes of membrane proteins. To address these and other issues, attempts have been made to produce improved SMA-related polymers. In this review we examine these new polymers in terms of performance.

SMALP self-assembly.

Much of the initial work on the use of SMA in membrane protein production focused on applications of SMA technology. These studies established the method as generically applicable to a range of proteins and showed that, once encapsulated, these proteins could be used in structural [12,23,24] and functional studies [9,13,25,26]. The subsequent increase in the use of the SMALP method has resulted in a number of studies about the mechanisms of SMALP self-assembly [18,27–31]. Scheidelaar *et al.* [27] have proposed a three-stage model for SMALP formation based on their experimental findings (Figure 1). First, SMA adsorbs to the surface of a phospholipid membrane (Figure 1a) in a process driven by the hydrophobic effect; direct interaction of styrene moieties of SMA with the acyl chains of lipids has been directly confirmed by NMR [23]. However, this behavior is also modulated by electrostatic repulsion, as indicated by increased solubilization in the presence of increased salt concentrations or lower amounts of anionic lipids [27]. In a second step, SMA buries into the hydrophobic acyl core of the membrane (Figure 1b). This stage has been demonstrated to be strongly dependent on lipid packing, with higher solubilization found above the Tm of the lipids, and maximal solubilization at Tm where the balance of gel phase and liquid crystalline phase results in packing defects, presumably allowing SMA to insert into these gaps. Finally, once the membrane is saturated with polymer and destabilised, SMALPs are formed (Figure 1c). SMALPs are stabilised in aqueous solution by the intercalation of phenyl groups of SMA between lipid acyl chains perpendicular to the plane of the bilayer, whilst the acid groups interact with the aqueous solvent, stabilizing the now solubilized phospholipid core (Figure 1c, inset) [23].

Influence of polymer composition on lipid particle generation.

In the case of classical detergents it is quite usual to find that only a subset of available detergents are able to successfully solubilise the membrane protein of interest. Therefore the process of choosing the correct detergent is often key to optimizing the extraction and purification of membrane proteins. By contrast, one benefit of the SMALP method is that the original SMA polymers were successful at solubilising a wide range of targets. However it would be complacent to think that the SMA polymers used in the early experiments represented the perfect solution, and it is undoubtedly this thought process that has led the development of new polymers. As more studies have investigated the SMALP self-assembly process, more insights have been gained about approaches to improve the process by modifying the chemistry of SMA copolymers. Figure 2 groups the modification of SMA-based copolymers into 4 categories: modification of the styrene to maleic acid ratio; modification of the hydrophobic component of the polymers; modification of the hydrophilic components and their subsequent functionalization; and finally, utilizing different polymerization methods to change the ordering of monomer units along the chain and size distribution of the polymers. Table 1 provides a summary of the properties of the different polymers that have been studied to date. The advances made in each of these areas will be discussed below.

Influence of the styrene:maleic acid ratio on polymer efficacy

In the first examples of SMA-mediated membrane protein solubilisations, two related SMA polymers were used with differing monomeric ratios: 2:1 S:M and 3:1 S:M. This points to an area where polymer chemistry may be modified: the ratio of styrene to maleic acid. By altering this ratio the hydrophobicity of the resulting polymer is altered, with 3:1 SMA being more hydrophobic than 2:1 SMA. One would therefore simplistically expect that 3:1 SMA would have a stronger thermodynamic driving force towards nanodisc self-assembly *via* interaction with phospholipid bilayers to bury the hydrophobic phenyl rings in the bilayer core rather than remaining dissociated in solution. This hypothesis has been confirmed by studies of the thermodynamics of disc formation by Keller *et al.* [29]. These studies have shown that the free energy change associated with SMA during SMALP self-assembly is more favourable for 3:1 SMA than 2:1. One may expect the thermodynamic efficiency of SMA polymers in SMALP self-assembly to directly relate to the solubilisation efficiency of membranes and proteins. However, the more relevant thermodynamic

parameter is the free energy change of lipids undergoing the vesicle to nanodisc transition. In all cases studied so far, the lipids have a small positive free energy change, although this is lower in 2:1 SMA [29] than 3:1 [32]. From a thermodynamic perspective, this indicates a more 'native-like' environment of the lipids within a nanodisc. Morrison *et al.* [30] have shown that the protein solubilisation efficiency of 3:1 or 2:1 SMA is broadly similar despite different thermodynamic efficiencies. However, Hall *et al*. [31] have demonstrated that a 2:1 SMA polymer with extended poly(styrene) hydrophobic stretches display poor protein solubilisation efficiency. Larger positive free energy changes were associated with the lipids undergoing SMALP formation, despite the high thermodynamic efficiency of the polymer. This suggests a disparity between polymers that appear to be thermodynamically efficient, yet are limited in their application to membrane protein extraction by a thermodynamically unfavourable lipid environment. Interestingly, a 1:1 SMA is also ineffective in membrane protein solubilisation [30]. This suggests that both a minimal level of hydrophobicity and an optimal distribution of hydrophobic moieties along the polymer chain influence the suitability of a given polymer for membrane protein extraction. Little work has been done to understand polymers that are substantially more hydrophobic (e.g. with a higher percentage of styrene) than the 3:1 polymer. Such a polymer would be interesting to study: although the increased hydrophobicity could aid solubilisation, it might also reduce the aqueous solubility of the polymer to such a level as to make the polymer ineffective. Without sufficient lipid, SMA polymers can form a collapsed higher order aggregate [31,33]. The structure of this aggregate is unknown, but it is likely to involve the partition of hydrophobic moieties into the interior of the aggregate, while the hydrophilic moieties mediate interactions with the aqueous solvent. The formation of SMALPs may be limited by the ability of polymer chains to dissociate from this aggregate before inserting into the target membrane. If this hypothesis is true then increasing hydrophobicity would stabilise the aggregate, thereby reducing the effectiveness of the polymer as a solubilisation agent.

Influence of the hydrophobic groups of the polymer.

While changing the S:M ratio alters hydrophobicity it is also possible to achieve the same effect by changing the hydrophobic monomer in the polymer. Major contributions in demonstrating the use of alternate hydrophobic moieties have come from work by Keller *et al.* [34,35]. These studies have shown that a related polymer (DIBMA, M_n 8.5 kDa, Figure 3)

containing aliphatic diisobutylene in place of aromatic styrene moieties is also functional in lipid particle self-assembly. Like SMA, DIBMA is capable of extracting proteins directly from cell membranes. The presence of a diisobutylene chain in place of styrene offers a number of advantages. The strong UV absorption of styrene overlaps with the UV absorption of intrinsic chromophores in membrane proteins (e.g. tryptophan, phenylalanine and tyrosine). The presence of diisobutylene in place of styrene allows for UV spectroscopic studies of membrane proteins solubilised using DIBMA, without absorption contributions from styrene in the polymer belt. In addition, calorimetry and Raman spectroscopy showed that the introduction of diisobutylene chains from DIBMA into the encapsulated lipid bilayer led to less perturbation of phospholipid bilayer dynamics in the lipid particle than phenyl rings from SMA. Jamshad *et al.* previously showed that phenyl rings from SMA inter-digitate into the bilayer in a similar fashion to cholesterol [23]. It is likely that this reduces molecular motions in the region of lipid bilayer near the polymeric annulus, forming a lipid raft-like rim in the membrane disc. Should this perturbation occur, it is likely that the physical properties of this region of lipid would be significantly different to that of a bulk phospholipid bilayer. This has the potential to affect the structure and function of any protein encapsulated in this region of the disc. At its most extreme, styrene groups could potentially interact directly with the encapsulated membrane protein, for instance inserting between α-helices inhibiting conformational changes in the protein. In contrast one might expect that diisobutylene chains in place of phenyl rings would provide a more native-like region at the polymer:lipid interface.

In addition, the DIBMA polymer is less sensitive than SMA to the presence of divalent cations. This is somewhat surprising as it was assumed that the maleic acid constituent of the polymer, which remains unchanged in DIBMA, mediated the interaction with divalent cations. These ions cause SMA to precipitate, limiting their use with SMALP-solubilised proteins, which is an issue particularly where such ions are an important element in bioassays. These observations make DIBMA seem an attractive alternative to SMA, however as the ability of DIBMA to solubilise a wide range of proteins yet to be proven. Nonetheless, the success of DIBMA in forming lipid particles also suggests a new range of functional groups for use as the hydrophobic moiety of the polymer. Further alterations in the chain-length of these polymers might provide more efficient than the existing polymers,

though attempts to form lipid particles from other maleic acid polymers with different hydrophobic monomers have been met with no success so far (Sandro Keller, personal communication, see Table 2). This suggests that there is a preference for certain chain geometries when forming stable lipid particles.

Influence of the hydrophilic groups of polymers

It is becoming apparent that changes can be made to the hydrophobic moiety in the polymer while retaining its disc-forming capability. The same question can be asked of the hydrophilic element of the polymer. There have been several reports where the maleic acid groups have been modified to an alternative hydrophilic moiety to produce a polymer which is functional in nanodisc formation. The route to modifying the polymer has, in general, been through modification of the anhydride form of a "parent" polymer in contrast to utilising alternate commercially available polymers with a different hydrophobic moiety. This has the advantage of preserving the underlying polymer architectures (e.g. length, dispersity and monomer ratios) that are known to be effective in SMALP self-assembly.

The first modification to the SMA backbone aimed to provide a wide range of possible functional chemical modifications to a single SMA polymer. Aubin *et al.* modified the anhydride form of SMA using cysteamine to add a sulphydril functional group (SMAnh-SH) before hydrolysis to the acid form (SMA-SH, M_n ND, Figure 3) [36]. Thiolation of SMAnh was performed at three molar ratios of cysteamine to SMAnh in the reaction in order to demonstrate that SMALP formation and size distribution is unaffected by different degrees of thiolation of SMA-SH. The addition of the sulphydril allows a wide range of common bioconjugation chemistries, which have been developed for modification of proteins, to be applied to the functionalisation of SMA-SH. Reagents developed to take advantage of free sulphydrils include dyes, affinity tags (e.g. Biotin), haptens, inorganics (e.g. Nanogold) and even antibodies. The presence of the sulphydril also provides the potential to crosslink the polymer in free form or as part of a lipid particle to another moiety. For example this could allow SMALPs containing proteins to be attached permanently to chromatography matrices or surfaces for application in techniques such as surface plasmon resonance (SPR). Other groups have built on this work and used the hydrolysed acid functional group to directly link

similar moieties to the SMA backbone [37], including using it as an alternative method for producing SMA-SH

Ravula *et al.* have also taken the approach of modification of a 'parent' polymer and shown that the substitution of *N*-(2-aminoethyl) moieties onto the anhydride group of SMAnh can be used to produce a positively charged polymer [38]. This new polymer, SMAd-A (Figure 3, Table 1) was made using a styrene maleic anhydride backbone with a M_n of 1.6 kDa, which is significantly lower than that for the conventional SMA 2:1 (2000, M_n 3.0 kDa) and 3:1 (3000, M_n 3.8 kDa) polymers used for the majority of protein solubilisations to date. Despite its smaller size, SMAd-A was effective in producing lipid particles when mixed with phospholipids (DMPC). Interestingly it was observed that altering the polymer-to-lipid ratio modulated the size of the discs, with larger discs being produced at higher lipid to polymer ratios. This observation agreed well with a previous study that shows a similar behavior for a 3:1 SMA polymer [39].

Our own unpublished work has shown how the maleic anhydride groups on the polymer can be substituted with dimethylaminopropylamine maleimide to form a positively charged polymer: poly(styrene-*co*-maleimide) (SMI, Figure 3). We have shown that despite this charge swap, SMI is still able to self-assemble in the presence of lipids into nanodiscs and the thermodynamics of this process are fairly similar to those measured for 2:1 SMA, 3:1 SMA and DIBMA. The size of the discs produced using this process is also similar to the other polymers, albeit slightly smaller. The major difference with SMI is that the pH range over which it functions is the reverse of that for maleic acid-based polymers. The SMI polymer produces lipid particles below pH 7.5 compared to above pH 6.5 for the negatively charged SMA polymers. Our data also shows that the positively charged polymer is much less sensitive to the presence of divalent cations than maleic acid-based polymers. This polymer will be vital in the production of membrane proteins whose properties complement these characteristics. For example membrane proteins that function at pH ranges below 7.5 (e.g. lysosomal proteins, certain acidophilic bacteria) would be more suited to solubilisation using this polymer. In addition the polymer could be used for proteins that required elevated levels of divalent cations for function (e.g. ATPases, ion-channels). Positive charge on the outside of the SMI-lipid particle, compared to the negative charge on the SMA-based polymers, could also be of importance in some applications. One potential problem with

positive charged polymers is that they are likely to interact with soluble biomolecules during the purification process, notably negatively charged proteins and DNA. It is therefore advisable that these polymers are used in buffers with a high ionic strength.

The presence of such a high charge density close to a protein may also influence on its function. Proteins such as cytochromes, for which electron transfer is an important part of their function, these areas of charge density could significantly influence protein activity. Proteins with a net surface charge that is complementary to that of the polymer annulus could experience considerable electrostatic attraction to the edge of the particle. Likewise, if the net protein charge matches that of the polymer belt the protein could become electrostatically confined to the center of the particle. In either case, this might not favour optimum protein function. It is also possible that the charge on the polymer may influence the areas of a biological membrane with which the polymer is able to interact, influencing its ability to solubilise proteins found in such regions. Once again, these effects should be mitigated by using buffers with high ionic strength.

Perhaps in response to these considerations, the hydrophilic element of the polymer has also been modified to produce a reagent with both positive and negative charges, namely a zwitterionic polymer. In one case, Fiori *et al*. replaced the carboxylic acid groups on SMA with cysteamine-phosphatidylcholine moieties to produce zSMA (Figure 3, Table 1) [40] while Ravula *et al.* substituted one of the carboxylic acid groups on each maleic acid moiety with *N*-(2-aminoethyl)amide to produce SMA-ED (M_n 1.6 kDa, Figure 3, Table 1) [38]. It is also important to note that the polymer scaffold on which SMA-ED is constructed is structurally closer than zSMA to the original SMA 2:1 polymer. Unlike zSMA, SMA-ED shares the same production chemistry for the polymer backbone (using the continually stirring tank reactor method), although it has a smaller mass (M_n 1.6 kDa, compared to 3.0 kDa for the SMA 2000). Analysis of the activity of the SMA-ED shows that it is as effective at solubilisation of DMPC membranes yielding discs that range in radius from approximately 4 to 10 nm in diameter depending upon the polymer:lipid ratio. The stability of lipid particles made using SMA-ED across a pH range showed that unlike SMALPs, the particles were stable at extremes of pH but between pH 6 and pH 4 the particles dissociated and aggregated. A separate study of the polymer alone showed similar behavior, suggesting that when both charged groups were ionized, intermolecular interactions occurred that led to polymer

dissociation from the lipid. The polymer was resistant to precipitation by divalent cations unlike SMA but this resistance only occurred when the carboxylic acid group was protonated at pH below 3.5. Unfortunately, this is incompatible with most membrane proteins.

The zwitterionic polymer made by Fiori *et al.* used a reversible addition-fragmentation chain transfer polymerization (RAFT)-synthesised SMA backbone that was modified with cysteamine-phosphatidylcholine [40]. As discussed later in this review, RAFT polymerization has a significant influence over the topology and size distribution of the polymers. Two zwitterionic SMA polymers (zSMA, Figure 3, Table 1) were produced with M_w values of approximately 21 kDa and 43 kDa. These were considerably larger than the SMA 2000-polymer used in most solubilisations, which has an M_w of 7.5 kDa. Studies of these polymers showed that each were proficient at solubilising membranes containing proteorhodopsin. It is interesting to note that the chemical group added in zSMA is not only zwitterionic but also significantly more bulky than the original maleic acid moiety. Estimations of the efficiency of extraction efficiency suggested that the zSMAs were as effective as conventional SMA 3:1 polymer. The important difference between conventional SMA and the zSMA variants was that, like DIBMA, zSMA showed much reduced susceptibility to divalent cations. Unlike SMA-ED this resistance occurred over the physiological pH range.

Influence of polymer architecture and size distribution

It is clear that the constituents of the polymer can have a significant influence on its solubilisation efficiency. These moieties are not the only aspect of a polymer that can be altered. Both the length of the polymer and the arrangement of monomeric units within the polymer sequence can also altered by changing synthesis conditions. In addition the dispersity of the polymer can be controlled. It should be forgotten that the vast majority of polymer preparations do not contain a single chemical entity but instead contain a statistical distribution of polymers. These polymers vary in length and composition around a mean value. Depending upon the synthesis conditions the variation from the mean can be adjusted producing polymers with high and low structural dispersities. For the SMA class of polymers the only aspect of the polymer sequence that cannot be easily altered concerns

the maleic anhydride constituent. The formation of a maleic acid to maleic acid bond (M-M) in the polymer is not possible in the polymerisation process (Figure 3b). Given this limitation a number of studies have examined the influence of polymer length on lipid particle formation. The first of these [30] examined a range of different SMA polymers made using the continually stirring tank reactor method (CSTR). This method is used to make the majority of commercially available polymers as it is easily deployed for the production of large quantities.

CSTR synthesis involves the active addition of reactants into a continuously running reaction to maintain a consistent polymer composition. Fully formed polymers are removed from the system throughout the reaction. This means, for example, that a polymer made using CSTR with parameters set for 3:1 styrene:maleic anhydride acid will contain statistically arranged styrene and maleic anhydride moieties in a 3:1 ratio. While CSTR produces polymers of relatively homogenous composition, the length of the polymer in the final product has a wider distribution than other methods that are discussed later. Analysis of the lipid particle formation of a range of these CSTR polymers [30] shows that the length of the polymer has a significant influence on whether the polymer is able to form a lipid particle. The size of the polymer also dictates whether that particle is stable enough to allow a protein-lipid-polymer particle to be captured and purified. These data show that extension of the polymer beyond M_n of 10 kDa yielded polymers that showed little or no ability to solubilise proteins from membranes.

It is unclear why increases in polymer length are so detrimental to membrane protein solubilisation efficiency. It could be that larger polymers are too long to form stable discoidal structures. To examine if this might be true a relatively simple analysis of polymer size can be carried out. This is somewhat naïve in concept as it only assumes an extended conformation of the polymer but it does give some insights into the scale of the polymer verses that of the disc. If one assumes that the SMA has an extended chain conformation when bound to the lipid particle then for each monomer with 2:1 S:M has an approximate length of 0.78 nm (3 times the distance between two terminal carbons in propane) when extended. If we assume a polymer with a M_n of 3.0 kDa, which is in line with the average mass of SMA 2:1 used in most solublisations, then a 2:1 ratio of would have 9 copies of the SSM unit. This means that the polymer has a length of 7 nm. Assuming a disc diameter of 10

nm, in line with most measurements of disc size, this makes the circumference 31.4 nm. Hence, five polymers of 3.0 kDa SMA 2000 are required to encircle an average-sized disc. This assumes one layer of SMA but it is possible that two layers of SMA are required to cover completely the edge of the disc. Polymers significantly larger than this may be too long to comfortably wrap around a 10 nm diameter disc without leaving a free tail. Presumably this unassociated polymer reduces the stability of the complex. From thermodynamic studies of SMALP self-assembly [29,31] the large negative free energy change associated with the polymer upon the vesicle to nanodisc transition indicates that the polymers interaction with lipids is favoured over self-interaction, supporting this hypothesis. Without more detailed structural information it is difficult to prove that such a simplistic analysis of polymer size is accurate in predicting the disc forming capacity of novel polymers. However the observation that longer polymers do not function as well as small polymers is suggestive that certain sizes of polymer are favoured. In interpreting these data, one caveat should be mentioned: studies have also shown that the polymer:lipid ratio can alter the size of discs formed. For example Ravula, Fiori, and Hall have all shown that increasing the lipid-to-polymer ratio leads to formation of larger discs [31,40,41]. Under the different conditions used in each study, it is difficult to understand how this effect is modulated by different polymers and will require further research to dissect these effects.

As already mentioned, the CSTR polymer production method provides good composition control but leads to a broad size distribution. In contrast, an alternative method of making polymers, reversible addition fragmentation chain transfer polymerization (RAFT), allows closer control of both size and polymer sequence. Smith *et al.* used this method to assess the effectiveness of a number of polymer architectures [42]. To create these different polymer species, RAFT polymerisation reactions were initiated under different starting conditions and samples taken at different stages in the polymerisation process. Based on simulations of the polymerisation developed by Smith *et al*., the depletion of monomeric maleimide as the reaction progresses increased the likelihood of longer stretches of styrene occurring in the polymer chain. If the reaction were allowed to progress to completion, all maleimide would have been incorporated in the chains, leading to the excess styrene forming a poly(styrene) tail. These studies showed that the styrene content of the polymer has a significant influence on lipid solubilisation capability. In

general, polymers made with styrene to maleic acid ratio exceeding 1.5:1 were more efficient at solubilising lipid.

Some influence of polymer length was also observed with a shorter version (RAFT-SMA A1, M_n 2.0 kDa) of a 1.94:1 ratio polymer being much less efficient at solubilisation than a longer version (RAFT-SMA A2, M_n 3.0 kDa). This might suggest that there is a lower limit for polymer size to be effective at generating lipid particles from a bilayer, although it should be noted that a polymer of similar mass (RAFT-SMA B1, M_n 1.8 kDa) but with a decreased styrene content (1.5:1 ratio) is an effective solubiliser of lipid. This suggests a complex interplay exists between polymer length and styrene-to-maleic acid ratio. The most effective polymer made using this method, RAFT-SMA D, was approximately 60% as effective as SMA 2000 made using the CSTR method. This polymer had a styrene to maleic acid ratio of 1.63:1 and a M_n of 2.8 kDa. Interestingly the architecture of this polymer included a short styrene-rich region at one terminus. Comparison of this RAFT-SMA D with similar RAFT-SMA (C2 or B3) synthesized with the same monomer ratio and chain length, but lacking a poly(styrene) tail, indicated that RAFT-SMA D is more effective. This may suggest that in this case the hydrophobic poly(styrene) tail could play a role in lipid particle formation. For example its hydrophobicity may mean that it forms the initial interaction between the polymer and the lipid bilayer. However one must be careful drawing too many conclusions from this experiment as the SMA 2000polymer (produced using CSTR) that was more effective at solubilising lipids has no tail. To further confirm that the presence of a hydrophobic tail may not be helpful, a RAFT-SMA with similar length and styrene to maleic acid ratio to the CSTR-synthesized 2:1 SMA was synthesized (RAFT-SMA E). Again this would have a tail containing a high styrene content compared to the CSTR polymer. These experiments showed that this polymer was only a third as effective as the SMA 2000 (2:1 produced using CSTR) polymer at solubilising lipids. This conclusion is supported by Hall *et al*. [31] who showed that a 2:1 RAFT-SMA with a poly(styrene) tail (M_n 6 kDa) is less effective in membrane protein solubilisation from *E. coli* membranes than SMA 2000. The authors attributed the decreased performance to the poly(styrene) tail burying into the bilayer core of the particle, potentially interacting with the solubilised membrane protein.

Smith *et al.* also examined how polymer length altered the size of the discs produced during lipid solubilisation. The most effective polymer produced using the RAFT process (D, M_n of 2.80 kDa and an S:M of 1.63:1) produced lipid particles that had a significantly smaller size distribution than the CSTR 2:1 (SMA 2000) polymer. This could have important influence on the use of this type of polymer. The increased structural homogeneity could have advantages for techniques such as small-angle scattering and cryo-EM, where sample homogeneity is important. However the reduced disc diameter may mean that a more limited number of proteins and their complexes can be solubilised. This correlates with observations from zSMA made using RAFT polymerisation that showed that polymers with M_n of 21.5 kDa and 43.7 kDa produced discs with diameters of approximately 17 and 35 nm respectively [40]. However it should also be noted that the shortest polymer of the sequence (M_n of 1.82 kDa) formed larger discs than any of the other polymers in the sequence. This is clear evidence of more than one shorter polymer interacting with the lipid generating a larger disc. The final observation made in this study was that the ability to solubilise pure lipid and lipid-containing protein was not necessarily linked. A set of polymers with styrene:maleic acid ratios close to 1.3:1 that were shown to be very ineffective solubilisers of pure phospholipids proved to be reasonably good solubilisers of membranes containing proteins. There are two possible explanations for this observation; either native lipids show more susceptibility to solubilisation, or the polymer is able to interact in a more favourable manner to the protein:lipid complex than lipids alone.

Conclusion.

The importance of membrane proteins in our understanding of biology means that studying their structure and function has to be an essential part of biochemistry as a discipline. This interest is magnified by the importance of membrane proteins in human disease and hence the development of new drugs and treatments. Despite this our studies of these proteins has lagged significantly behind other biomolecules, largely because it is so difficult to produce stable active samples of membrane proteins.

The development of the SMALP method for the solubilisation of membrane proteins in 2007, for the first time, provided a protocol that allowed membrane proteins to be extracted with their local lipid environment intact [9]. This has allowed samples of a number of membrane proteins to be generated that retain function and have substantially enhanced

stability. However our enthusiasm for extracting membrane proteins using this method has exceeded our understanding of the process itself, and more specifically, our understanding of the influence of the polymer on the process. In this review we have drawn together a number of independent studies that have examined the use of a range of polymer variants in the formation of SMALPs and SMALP-like particles. These studies have shown that the length and composition of the polymers used to form lipid particles can have significant influence over the size and stability of the assembly. There appears to be an optimal balance of hydrophobic and hydrophilic character within the polymer exemplified by a range between 1.5:1 and 3:1 S:M ratio. In addition there seems to be general agreement that polymers with sizes below M_n of 5 kDa offer the optimum performance in the formation of lipid particles. The polymer sequence has also been shown to influence activity; polymers without a styrene tail are in general the most effective solubilisers.

The introduction of different hydrophobic and hydrophilic monomers has shown that the overall approach is surprisingly rugged, with a wide range of substituents supporting lipid particle formation. This has allowed a range of polymers with different physical properties to be developed that will have applications in areas where the existing SMA polymers do not perform (e.g. at low pH and high divalent cation concentrations).

Finally the development of polymers that allow sporadic functionalisation (e.g. SMA-SH) provide the exciting opportunity to generate reagents for specific downstream applications, including polymers that bind to specific resins or surfaces or that can be tracked optically.

All of these developments are substantially increasing the reagent “tool kit” available to the study of membrane proteins as well as providing a simpler alternative to the historical detergent screening process. For example, polymers forming larger discs would be ideal for the solubilisation of large membrane proteins and complexes. Increased structural homogeneity of the resultant nanodiscs would be ideal for downstream applications such as small angle scattering and the rapidly evolving field of cryo-TEM. Likewise, if low pH or the presence of divalent cations is required for function or stability of the membrane protein of interest, polymers have now been developed that can tolerate these solution conditions. .

However, a number of caveats should be noted in response to the development of new lipid disc-forming polymers. Firstly, the majority of these polymers have not been tested on a wide range of biological membranes. Secondly, it will take some time to discover

whether these new polymers are applicable in the production of a broad range of proteins. Lastly, the growth in the number of polymers means that it may be more difficult to choose the ideal reagent for all downstream applications, leading to the requirement for polymer screening. Nonetheless, the advantages of certain polymer variants cannot be disputed, and development of further variants with optimized properties for given applications should be encouraged. With this comes the necessity for consistent comparisons to known and widely used polymers to facilitate the process of choosing the best performing polymer for the desired application. This approach has been successfully adopted in recent studies [30,36,42] and, if continued, will lead to more widespread adoption of the exciting new polymers that are being developed.

It is clear from this review that the success of the SMALP method has generated a lot of interest in the research community, which has also catalysed the development of new and improved lipid disc-forming polymers. This can only go to ensure that the production of membrane proteins using the SMALP method will grow in success and acceptance. The generation of new polymers has certainly opened a wide variety of additional routes to help in the production of membrane proteins. However time will tell whether these new reagents will perform better than established polymers. In particular it will be interesting to see whether these new polymers address some of the persistent issues with SMALP methods including perhaps the most important issue, the preservation of conformational changes in encapsulated membrane proteins.

FIGURES.

Figure 1. Schematic representation of the 3-stage SMALP self-assembly process. **a**. SMA (green ribbons) chains adsorb to the phospholipid bilayer surface. **b**. SMA chains bury into the acyl core of the bilayer until the membrane is saturated with polymer. **c**. As the membrane becomes saturated with polymer, it becomes destabilised, allowing for SMALP formation. SMALP nanodiscs are stabilised by burying of phenyl groups of SMA into the acyl core of the phospholipid bilayer, while acid groups allow interaction with the aqueous solvent.

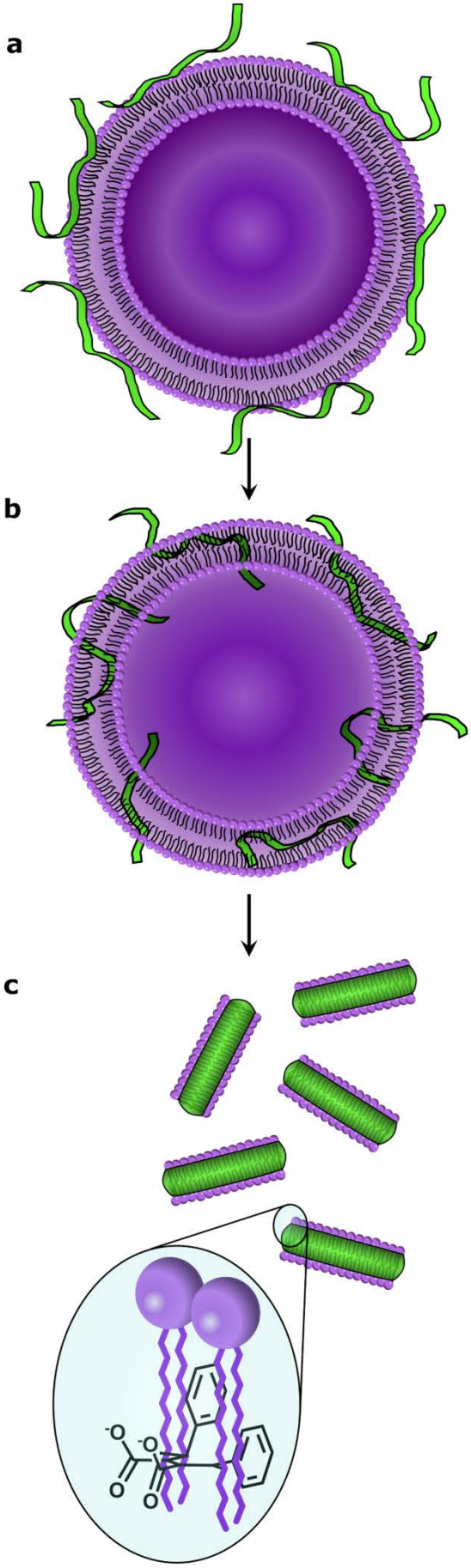
a
b
c

Figure 2. The four modes of variation for SMA and SMA-related polymers used for lipid particle production. a) A diagrammatic representation of a generic SMA polymer. Orange circles represent the hydrophobic styrene groups and blue circles represent the hydrophilic, negatively charged maleic acid groups. b) Changing the monomer ratio. The most common variants tested have consisted of either a 1:1, 2:1 or 3:1 styrene:maleic acid ratio. These can either be strictly alternating or stastistically arranged monomer units, though synthesis conditions prohibit the formation of maleic acid dimers. c) Modification of the hydrophobic groups. Aromatic phenyl rings can be replaced by branched aliphatic chains. d) Modification of the hydrophilic groups. The hydrophilic groups can be modified in two ways, either by complete substitution of maleic acid during synthesis to yield polymers of differing charge, or by functionalisation of an SMA polymer to add additional functional groups. e) Utilising alternative synthesis methods to generate polymers of differing architectures and size distributions. Continually stirring tank reactor (CSTR) synthesis yields statistically arranged monomer units along the polymer whilst giving a broad size distribution as measured by the polydispersity index. Reversible addition-fragmentation chain transfer (RAFT) polymerization yields alternating polymers with an extended hydrophobic tail, with a much more narrow size distribution.

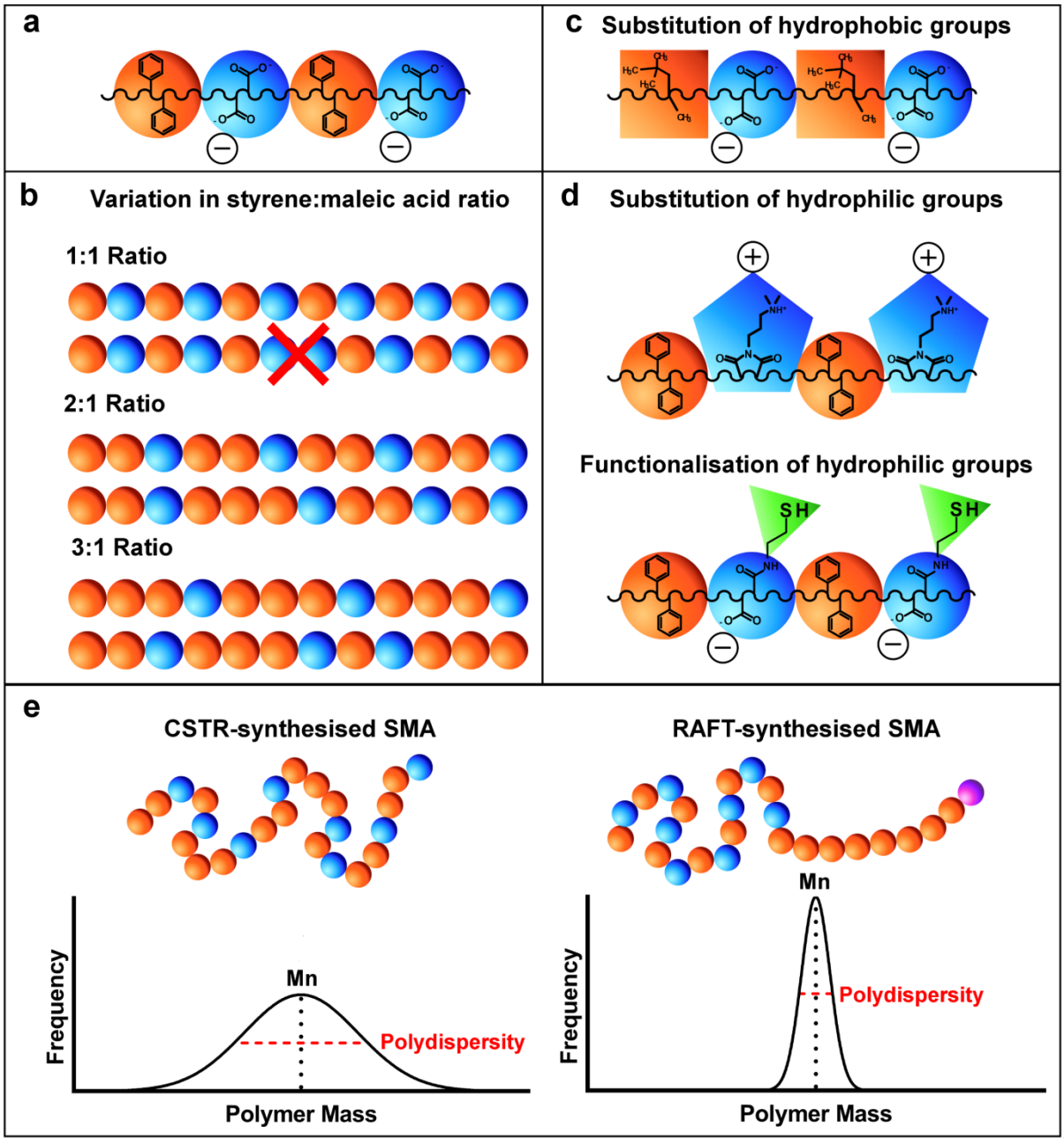
a
c Substitution of hydrophobic groups
b Variation in styrene:maleic acid ratio
1:1 Ratio
2:1 Ratio
3:1 Ratio
d Substitution of hydrophilic groups
Functionalisation of hydrophilic groups
SH
SH
e
CSTR-synthesised SMA
RAFT-synthesised SMA
Mn
Polydispersity
Frequency
Polymer Mass
Mn
Polydispersity
Frequency
Polymer Mass

Figure 3. Chemical structures of different SMA polymer derivatives used for membrane protein encapsulation. The polymers have been categorised according to the synthesis method. Hydrophobic and hydrophilic functional groups are coloured red and blue, respectively. Modifications made to the hydrophilic groups have been coloured green. Purple functional groups correspond to the end-group present on the polymer due to the choice of RAFT agent used during polymer synthesis.

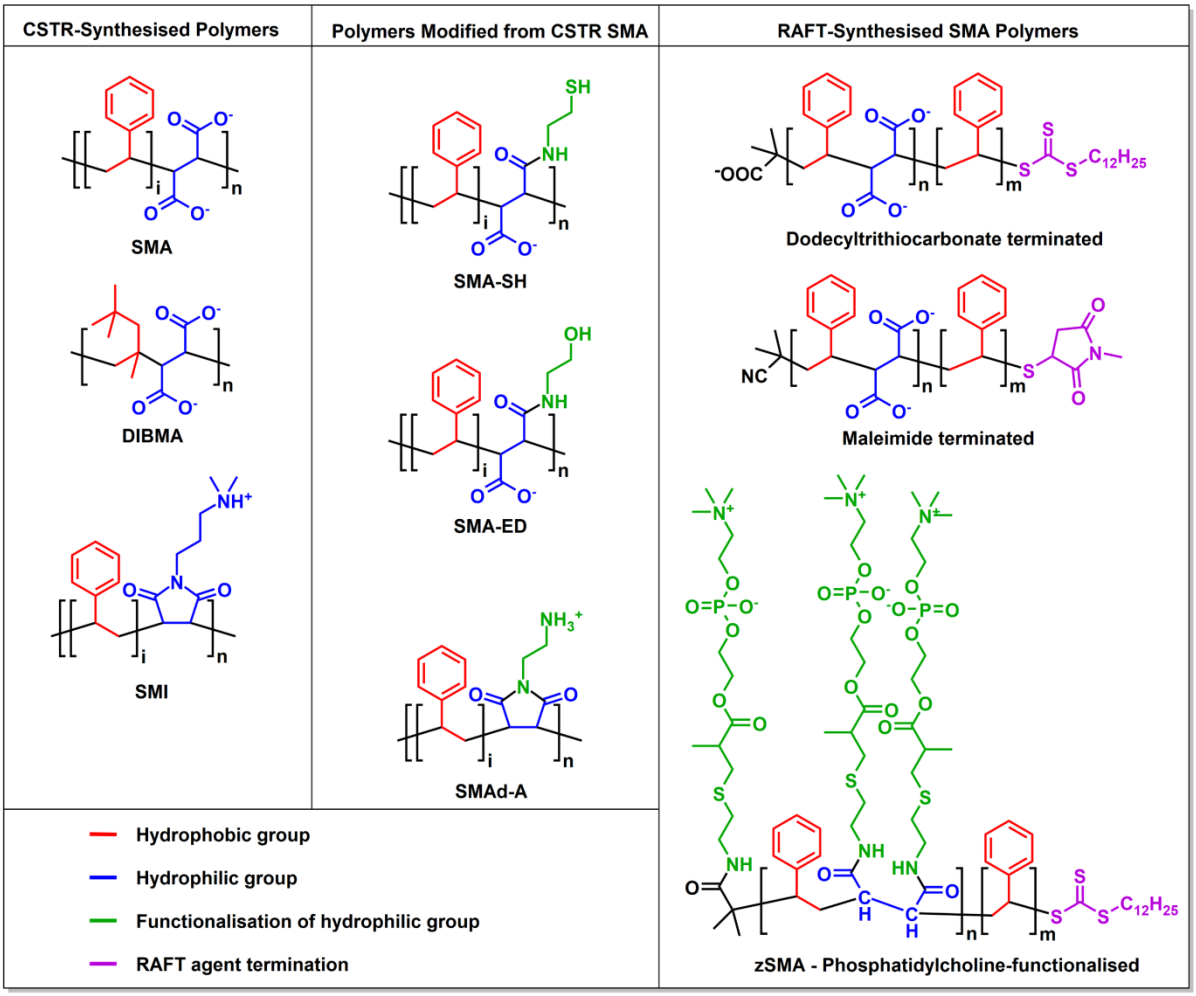

TABLE 1. Summary of SMA and SMA-like polymers used for the production of lipid particles. Where available, details of the lipid particles produced by each polymer are included. Qualitative assessments of lipid and membrane protein solubilisations are made with reference to solubilisation using SMA 2000.

Polymer Name	**H-phobic**	**H-philic**	**% MA**	**Secondary Functional-isation**	**Mw (g/M)**	**Mn (g/M)**	**Method**	**Disk Size**	**pH Range**	**Ions**	**Solubilisation Lipid**	**Lipids investigated**	**Solubilisation Protein**	**Proteins investigated**
SMA 1000	Styrene	Maleic Acid	50		5500	2000	CSTR		Only pH 8 published				1	LeuT, ZipA, BmrA [30]
SMA 2000	Styrene	Maleic Acid	33		7500	3000	CSTR	10	> pH 7.5	< 4 mM $MgCl_2$ or Ca	3	Examples include DMPC, POCP/POPE [32]	3	Examples include bacteriorhodopsin, PagP [9] LeuT, ZipA, BmrA [30]
SMA 3000	Styrene	Maleic Acid	25		9500	3800	CSTR	5 (DLS)	Only pH 8 published	$MgCl_2$ not tolerated	Successful - No comparison to SMA2000	Native mitochondrial membranes [22]	2	Examples include SecYEG [43] LeuT, ZipA, BmrA [30]
XZ09006	Styrene	Maleic Acid	40		7500		CSTR		Only pH 8 published	< 4 mM $MgCl_2$			2	LeuT, ZipA, BmrA [30]
XZ09008	Styrene	Maleic Acid	25		10000		CSTR	5 (DLS)	Only pH 8 published	$MgCl_2$ not tolerated			3	

SZ40005	Styrene	Maleic Acid	42		5000	2000	CSTR		Only pH 8 published			1	
SZ25010	Styrene	Maleic Acid	25		10000		CSTR	5 (DLS)	Only pH 8 published	$MgCl_2$ not tolerated		3	
SZ42010	Styrene	Maleic Acid	42		10000		CSTR		Only pH 8 published			1	
SZ33030	Styrene	Maleic Acid	33		30000		CSTR		Only pH 8 published			1	
SZ28065	Styrene	Maleic Acid	28		65000		CSTR		Only pH 8 published			1	
SZ28110	Styrene	Maleic Acid	28		110000		CSTR		Only pH 8 published			1	
SZ30010	Styrene	Maleic Acid	31		10000	2500	CSTR		Only pH 8 published			3	*Rhodobacter sphaeroides* reaction centres (RCs) [44]
SZ30030	Styrene	Maleic Acid	33		30000	9000	CSTR		Only pH 8 published			3	
SZ26030	Styrene	Maleic Acid	24		10000	4000	CSTR		Only pH 8 published			3	
SZ26080	Styrene	Maleic Acid	25		80000	32000	CSTR		Only pH 8 published			2	
SZ26120	Styrene	Maleic Acid	25		120000	48000	CSTR		Only pH 8 published			2	
SZ20010	Styrene	Maleic Acid	19		11000	25000	CSTR		Only pH 8 published			0	
DIBMA	Diisobutylene	Maleic Acid			15000	8500	CSTR	30 (DLS)	pH 7.4 and 8.3	At least 20 mM Ca^{2+} or Mg^{2+}	3	DLPC, DMPC, DPPC, POPC [36]	3	OmpLA and range of MPs by SDS-PAGE [34]

SMA-SH	Styrene	Maleic Acid	33	Cysteamine	7500	Not published	CSTR	10 nm (TEM DLS)	Only pH 8 published	Not published	3	DMPC [36]	3	Bacteriorhodopsin [36]
A1	Styrene	Maleic Acid	5	Terminal malemide	1200	2000	RAFT		Published pH 7.4		1	Liss Rho PE with DOPC [42]		
A2	Styrene	Maleic Acid	5	Terminal malemide	2200	3000	RAFT				2			
A3	Styrene	Maleic Acid	5	Terminal malemide	7600	9100	RAFT				2			
B1	Styrene	Maleic Acid	10	Terminal malemide	1500	1800	RAFT				2			
B2	Styrene	Maleic Acid	10	Terminal malemide	3700	2300	RAFT				2			
B3	Styrene	Maleic Acid	10	Terminal malemide	4200	3300	RAFT				2			
B4	Styrene	Maleic Acid	10	Terminal malemide	5100	5100	RAFT				2			
B5	Styrene	Maleic Acid	10	Terminal malemide	7100	7000	RAFT				1		0	Oligomeric membrane protein [42]
C1	Styrene	Maleic Acid	20	Terminal malemide	1700	1400	RAFT				1		2	
C2	Styrene	Maleic Acid	20	Terminal malemide	3700	2500	RAFT				1	Liss Rho PE with DOPC [42]	2	
C3	Styrene	Maleic Acid	20	Terminal malemide	4200	3900	RAFT				1		2	
C4	Styrene	Maleic Acid	20	Terminal malemide	4800	4100	RAFT				1		2	
C5	Styrene	Maleic Acid	20	Terminal malemide	5900	5000	RAFT				1		0	

D	Styrene	Maleic Acid	25	Terminal malemide	3100	2900	RAFT				3		3	
E	Styrene	Maleic Acid	33	Terminal malemide	3800	3400	RAFT				2			
F	Styrene	Maleic Acid	25	Terminal malemide	7000	5400	RAFT				2		0	Oligomeric membrane protein [42]
HPBD-b-$(P4VP2_8)_2$	Poly(4-vinylpyridine)	Hydrogenated polybutadiene		Encased in MSP1E3D1	1000	9000	RAFT	11 (DLS)	pH 7.5		Successful - No comparison to SMA2000	*E. coli* polar lipid 42]	Successful - No comparison to SMA2000	MsbA [45]
SMA-ED	Styrene	Maleic Acid	57	Ethylene-diamine		1600	CSTR	5-10	Stable at pH < 5 and > 7	< 200 mM Ca^{2+} and Mg^{2+} at pH 3.5. No tolerance at pH 8.5	Successful - No comparison to SMA2000	DMPC [38]		
SMAd-A	Styrene	Maleic Acid	57	Dehydrated SMA-ED		1600	CSTR	3-10	Stable at pH < 6	< 200 mM Ca^{2+} and Mg^{2+} at pH 3.5.	Successful - No comparison to SMA2000	DMPC [38]		
SMA-EA	Styrene	Maleic	57	Ethanolami		1600	CSTR				Succes	DMPC		

		Acid		ne							ful - No comparison to SMA2000	[41]		
zSMA1	Styrene	Maleic Acid		Phosphatidylcholine	12,500	ND	RAFT	10 nm (DLS)	pH 5 and 8	< 5 mM Mg or $CaCl_2$	3	E. coli total lipid [40]	3	MsbA [40]
zSMA2	Styrene	Maleic Acid		Phosphatidylcholine	21,500	35,000	RAFT	17 nm (DLS)			Successful - No comparison to SMA2000		Successful - No comparison to SMA2000	
zSMA3	Styrene	Maleic Acid		Phosphatidylcholine	43,800	53,000	RAFT	30 nm (DLS)			Successful - No comparison to SMA2000		Successful - No comparison to SMA2000	

Table 2. A list of polymers that have been unsuccessfully trialled for lipid particle production (personal comm. Dr Sandro Keller)

Polymer name	**Polymer Composition**	**Mw (g/mole)**	**Lipid tested**	**Supplier**
Nvoy	Chemical structure not revealed by manufacturer	5000	POPC	Expedeon
No trade name supplied	Poly(isobutylene-*alt*-maleic anhydride)	6000	POPC	Sigma

EUDRAGIT® E 100	Poly(butyl methacrylate-*co*-(2-di-methylaminoethyl) methacrylate-*co*-methyl methacrylate)	47000	POPC	Evonik
EUDRAGIT® E PO	Poly(butyl methacrylate-*co*-(2-di-methylaminoethyl) methacrylate-*co*-methyl methacrylate)	47000	POPC	Evonik
EUDRAGIT® L 100	Poly(methacrylic acid-*co*-methyl methacrylate)	125000	POPC	Evonik
EUDRAGIT® S100	Poly(methacrylic acid-*co*-methyl methacrylate)	125000	POPC	Evonik
Gantrez AN-119	Poly(methyl vinyl ether-*co*-maleic acid)	130000	DMPC	Ashland
EUDRAGIT® FS 30 D	Poly(methyl acrylate-*co*-methyl methacrylate-*co*-methacrylic acid)	280000	POPC	Evonik
EUDRAGIT® L 30 D-55	Poly(methacrylic acid-*co*-ethyl acrylate)	320000	POPC	Evonik
EUDRAGIT® L 100-55	Poly(methacrylic acid-*co*-ethyl acrylate)	320000	POPC	Evonik
EUDRAGIT® NE 40 D	Poly(ethyl acrylate-*co*-methyl methacrylate)	750000	POPC	Evonik

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